REMARKS

Pursuant to MPEP 706.07(h), on filing an RCE, Applicant may direct that the previously filed, but un-entered After-final amendment not be entered, but that, instead, the present submission be entered for consideration by the Examiner. Accordingly, Applicant requests that the previously filed After-final amendment (filed on September 2, 2004) not be entered into the file and request entry of the instant amendments and remarks.

Claims 64-94 are currently pending in the application. Claims 65, 67-70, 71-74, 76 and 86 are amended. Claim 69 is cancelled. The amendments are supported in the specification as specifically discussed below. Claims 88-94 are added. The added claims 88-94 are supported throughout the specification, e.g., on pages 27-29 (Tables 2A and 2B), and claim 88 is also supported in previous claim 74 before its amendment. No new matter is added.

Amendments to the Specification

Table 2B on page 28 is amended to correct clerical errors regarding the position of specific mutations for the Vent and Deep Vent DNA polymerases. The amended positions are supported in Table 2B as originally filed. No new matter is added. Applicants respectfully request the entry of the amendments.

In a telephone interview of June 21, 2004, Applicants and the Examiner has agreed to incorporate the sequences for JDF-3 DNA polymerase into the present specification, although Applicants believe the specification as originally filed (i.e., without the incorporation) already satisfies the enablement requirement.

Such incorporation is supported under MPEP 608.01(p). Applicants respectfully request the entry of the incorporation.

Claim Objection

Claim 72 is objected to because of the recitation of "mutant KDO DNA polymerase." Applicants have corrected the clerical error to recite "mutant KOD DNA polymerase." Applicants respectfully request the withdrawn of the claim objection on claim 72.

Claim Rejections under 35 U.S.C. §112, Second Paragraph

Claims 69-74 are rejected under 35 U.S.C. §112, second paragraph. The Office Action states that claims 69-74 are indefinite because it is not clear the extent to which the genus if further limited. The Office Action states:

> "The problem is that claim 69 makes no limitation that the claimed mutant polymerase must be a mutant Pfu DNA polymerase thus in effect claim 69 is drawn to the enzyme mixture of claim 67, wherein said mutant DNA polymerase comrpising a mutation in its partitioning domain or polymerase domain is a mutant Pfu DNA polymerase, KOD DNA polymerase, or JDF-3 DNA polymerase, wherein said mutant DNA mutant Pfu DNA polymerase contains a mutation at an amino acid position selected from the group consisting of at D405, Y410, T542, D543, K593, Y595, Y385, G387 or G388. Thus claim 69 still encompasses the specific amino acid mutants of Pfu as well as partitioning domain or polymerase domain mutants of KOD DNA polymerase, or JDF-3 DNA polymerase."

Applicants respectfully disagree. Applicants submit that the claims as written were clear as to the extent that they further limit claim 68. However, for the sole purpose of expediting prosecution, Applicants have amended claims 69, 71-74. Claims 69, 71-74 are amended. The amendment finds support on pages 27-29 (Tables 2A and 2B).

Applicants submit that the above claim amendments obviates the 35 U.S.C. 112, second paragraph rejections on claims 69-74. Applicants respectfully request the withdrawn of the indefiniteness rejections on claims 69-74.

Claim Rejections under 35 U.S.C. §112, First Paragraph

Claims 65, 66, 68-69, 73-74, 76-81 and 83-84 are rejected under 35 U.S.C. §112, first paragraph. The Office Action states that these claims are not enabling and that a deposit of the referred bacterial DNA polymerases is required.

Applicants respectfully disagree. In an interview with Applicants' representatives on June 21, 2004 (see Statement of Substance submitted herewith), Examiner Hutson clarified that the enablement rejection was issued in particular because of the recitation of the JDF-3 DNA polymerase. The Examiner stated a deposit for JDF-3, not for any other DNA polymerases recited in the claims, was required because JDF-3 DNA polymerase was not deemed readily accessible to the public.

Applicants respectfully disagree. Applicants submit that all DNA polymerases recited which can be used as the first enzyme of the present invention, including the JDF-3 DNA polymerase, are known in the art and they are readily accessible to the public. No deposit for any of the DNA polymerases is required.

First, Applicants submit that all recited DNA polymerases are well known in the art. As stated in the previous response filed December 18, 2003, the specification provides sequence accession number for each of the claimed DNA polymerase (e.g., pages 14-19). The specification further provides at least one publication reference for each of the DNA polymerases recited in the rejected claims (e.g., on page 12).

Second, Applicants submit that the recited DNA polymerases are readily accessible to the public. It is routine for one skilled in the art of molecular biology to express a protein based on its known nucleotide or amino acid sequence. The instant specification specifically teaches the expression and purification of a DNA polymerase (mutant or wild-type) using a polynucleotide encoding the DNA polymerase (e.g., pages 33-34, Example 2). No undue experimentation is required for such routine expression and purification of any of the DNA polymerases recited.

In addition to the teachings of the specification, many of the DNA polymerases were commercially available and their availability was known to one skilled in the art. For example, page 16 of the present specification provides the availability of some DNA polymerases from various commercial sources. Applicants herein further provide more detailed information on the commercial availability of the DNA polymerases:

DNA polymerases	**Vendor**	**Catalog #**
Taq DNA polymerase	Stratagene, La Jolla, CA	600131, 600132, 600139
	Promega, Madison, WI	M1661, M1665, M1668, M1861, M1865
Tth DNA polymerase	Promega, Madison, WI	M2101, M2105
Tli (Vent) DNA polymerase	New England Biolabs, Beverly, MA	254S
	Promega, Madison, WI	M7101
Tgo DNA polymerase	Roche Applied Science, Indianapolis, IN	3186172
Pfu DNA polymerase	Stratagene, La Jolla, CA	600135, 600136, 600140,
	Promega, Madison, WI	M7741, M7745
KOD DNA polymerase	Novagen, San Diego, CA	71085-3
PGB-D (Deep Vent) DNA polymerase	New England Biolabs, Beverly, MA	258S, 258L
Pwo DNA polymerase	Boehringer Mannheim, Indianapolis, IN	1644947

Third, Examiner Hutson clarified during the interview that no deposit was required for any other DNA polymerases, but the claims were rejected for their recitation of "JDF-3 DNA

polymerase." The Examiner felt that the JDF-3 DNA polymerase was not readily accessible to the public.

With respect to **JDF-3 DNA polymerase** particularly, Applicants submit that JDF-3 DNA polymerase was not only known in the art, but also readily accessible to the public as of the instant patent application filing date. For example, JDF-3 DNA polymerase is described on page 12 (lines 19-20 During our June 21, 2004 telephone interview, Examiner Hutson agreed with Applicants' that JDF-3 polypeptide sequence was available, but maintained that the phrase "JDF-3 DNA polymerase," as used in the claims, even in view of the teachings in the specification, did not specifically disclose the particular JDF-3 sequence which would enable the making and using of the JDF-3 DNA polymerase in the claimed invention. Although Applicants believe the above teachings satisfy the enablement requirement for JDF-3 DNA polymerase, for the sole purpose of expediting the prosecution, Applicants thereby further incorporate JDF-3 amino acid sequence (SEQ ID NO:2 of WO 01/32887) and its corresponding DNA sequence (SEQ ID NO:1 of WO 01/32887) into the present specification. The incorporation of the sequences is permitted under MPEP 608.01(p). Examiner Hutson agreed that such incorporation would obviates the enablement rejections on claims 65-66, 68-69, 73-74, 76-81 and 83-84 because of the recitation of JDF-3 DNA polymerase. During the interview of June 21, 2004, Applicants' representative, by mistake, referred to US Patent No. 5,602,011 recited on page 16 of the specification as the patent that contains the JDf-3 sequences. Applicants wish to correct the mistake and state that WO 01/32887 recited on page 12 of the present specification contains the correct JDF-3 polypeptide and nucleotide sequences.

In view of the above, Applicants submit that all DNA polymerases recited in claims 65-66, 68-69, 73-74, 76-81 and 83-84 are known in the art and are readily accessible to the public and/or they can be obtained without undue experimentation. One skilled in the art, therefore, will know how to make and use the present invention as claimed based on the teaching of the present specification. Applicants, therefore, respectfully request the lack of enablement rejections under112, first paragraph over claims 65-66, 68-69, 73-74, 76-81 and 83-84 be withdrawn.

Claim Rejections under 35 U.S.C. §103(a)

Claims 64-69, 75, 82-83 and 85-87 are rejected under 35 U.S.C. §103(a). The Office Action states that the claims are obvious over Barnes et al. (U.S. Patent No. 5,436,149) and Komori et al.

Applicants respectfully disagree based on the same reasoning presented in the previous response filed December 18, 2003.

Claims 64-66, 75 and 85

With respect to claims 64-66, 75 and 85, Applicants submit that these claims are drawn to an enzyme mixture containing a first enzyme and a second enzyme, *wherein said **first enzyme is an Archaeal DNA polymerase**, said second enzyme is a mutant **Archaeal DNA polymerase with a 3'-5' exonuclease activity** and a reduced DNA polymerization activity*. Therefore, the first enzyme is limited to an Archaeal DNA polymerase and the second enzyme is limited to an enzyme with a 3'-5' exonuclease activity (exo^+). As known in the art, all archaeal DNA polymerases contain 3'-5' exonuclease activity (e.g., See Exhibit A, page 327 and Figure 4, relevant text highlighted). Therefore, both the first and the second enzymes of the enzyme mixture as claimed in claims 64-66, 75 and 85 contain the 3'-5' exonuclease activity. That is, the enzyme mixture of the invention as claimed in 64-66, 75 and 85 comprises **two exo^+ enzymes**.

First, neither Barnes et al. nor Komori et al. teaches or suggest the present invention as claimed in claims 64-66, 75 and 85. Barnes et al. describes a formulation with a **majority DNA polymerase** component **lacking 3'-5' exonuclease activity** (exo^-, e.g., Taq DNA polymerase) and a **minority DNA polymerase** component **exhibiting 3'-5' exonuclease activity** (exo^+, e.g., wild type Pfu DNA polymerase). Therefore, the formulation in Barnes et al. comprises an exo^- enzyme **and** an exo^+ enzyme. In contrast to the teachings in Barnes et al., claims 64-66, 75 and 85 of the present invention claim an enzyme mixture comprising a first enzyme and a second enzyme, wherein the first enzyme is an Archaeal DNA polymerase (**one exo^+**) and said second enzyme is a mutant Archaeal DNA polymerase comprising a 3'-5' exonuclease activity and a

reduced DNA polymerization activity (**another exo$^+$**). Barnes et al. does not teach or suggest an enzyme mixture of two exo$^+$ enzymes. Komori et al. does not teach or suggest such an enzyme mixture either.

Second, there is no suggestion or motivation to combine the prior art references. In order to establish a prima facie case of obviousness, there must be some reason, suggestion, or motivation from the prior art as a whole that indicates that the person of ordinary skill would have combined or modified the references. The Federal Circuit has stated:

> "[O]bviousness cannot be established by combining the teachings of the prior art to produce the claimed invention, absent some teaching, suggestion or incentive supporting the combination."[1]

As stated above, Barnes et al. teaches a formulation of one exo$^-$ DNA polymerase and another exo$^+$ DNA polymerase. The formulation is provided based on the theory that the use of a DNA polymerase exhibiting *3'-exonuclease activity* (exo$^+$) can overcome the problem of a DNA polymerase lacking *3'-exonuclease activity* (exo$^-$), e.g., Taq DNA polymerase. Barnes et al. provides:

> "As speculated in Barnes (1992; supra), Thermus aquaticus DNA polymerase and its variants are slow to extend a mismatched base pain (which they cannot remove since they lack any 3'-exonuclease. A couple of companies (New England Biolabs and Stratagene) have introduced thermostable enzymes which exhibit a 3'-(editing) exonuclease which should, one would think, allow the removal of mismatched bases to result in both efficient extension and more accurately copied products. In practice, these two enzymes (Vent and Pfu DNA polymerase) are unreliable and much less efficient than expected....
>
> I have discovered that the expected beneficial effects of a 3'-exonuclease can be obtained with an unexpectedly minute presence of an Archaebacterial DNA polymerase, whilst efficient extension is being catalyzed by a large amount of (3'-exonuclease-free) KlenTaq-278 or AT." (Columns 16-17).

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[1] *In re Geiger*, 815 F.2d 686, 688, 2 U.S.P.Q.2d 1279, 1278 (Fed. Cir. 1987)

As one can see, Barnes specifically teaches a combination of *an exo$^-$ and an exo$^+$ DNA polymerases* so that *the presence of the exo+ DNA polymerase increases the amplification efficiency of the exo$^-$ DNA polymerase in the mixture.* Barnes et al. do not teach or suggest a enzyme mixture of an exo$^+$ DNA polymerase with another exo$^+$ DNA polymerase with reduced polymerization activity. In fact, if the first enzyme is an Archaeal DNA polymerase which already possesses the 3'-5' exonuclease activity, as claimed in claims 64-66, 75 and 85 of the present invention, there would be no motivation for one skilled in the art to mix the first enzyme with another exo$^+$ enzyme based on the teachings of Barnes et al. Therefore, it would not be obvious for one skilled in the art, in the absence of the present teaching, to make an enzyme mixture comprising a first and a second enzyme, where both enzymes contain 3'-5' exonuclease activity as claimed in claims 64-66, 75 and 85 of the present invention.

Komori et al. studies the structure-function relationship of Pfu DNA polymerase, i.e., what mutations affect or abolish the DNA polymerase and exonuclease activities of Pfu. Komori et al. does not teach or suggests that these two mutants can be used with another DNA polymerase, let alone be used with another exo$^+$ DNA polymerase (Archaeal DNA polymerase) as claimed in the present invention.

Therefore, there is no teaching or suggestion to combine or modify Barnes et al. and Komori et al. to reach to the present invention, that is, an enzyme mixture comprising a first enzyme and a second enzyme, wherein the first enzyme is an Archaeal DNA polymerase and the second enzyme is a mutant Archaeal DNA polymerase comprising 3'-5' exonuclease activity and a reduced DNA polymerization activity. Because the prior art references fail to provide any suggestion or incentive to combine or modify the references, the Office Action fails to establish a prima facie case of obviousness.

Third, even when the prior art references are combined, they do not result in the present invention as claimed in claims 64-66, 75 and 85. The combination of Barnes et al. and Komori et al. still does not teach or suggest an enzyme mixture comprising a first enzyme and a second enzyme, wherein the first enzyme is an Archaeal DNA polymerase and the second enzyme is a

mutant Archaeal DNA polymerase comprising 3'-5' exonuclease activity and a reduced DNA polymerization activity, that is, both enzymes are exo^+ enzymes.

In view of the above, Applicants submit that neither Barnes et al, or Komori et al., alone or in combination, teach or suggest the invention as claimed in claims 64-66, 75 and 85 of the present invention, i.e., to make an enzyme mixture comprising a first enzyme and a second enzyme, where the *first enzyme is an Archaeal DNA polymerase, said second enzyme is a mutant Archaeal DNA polymerase with a 3'-5' exonuclease activity and a reduced DNA polymerization activity.*

Claims 67-69, 82-83, 86-87

With respect to other claims rejected under 103(a), namely, claims 67-69, 82-83, 86-87, Applicants submit that claims as previously presented are not obvious over Barnes et al. and Komori et al. However, for the sole purpose of expediting the present prosecution, Applicants have amended the claims. Applicants preserve the right of pursuing the subject matters as previously presented in claims 67-69, 82-83, 86-87 (i.e., D405 mutants) in a subsequent continuation application.

First, neither Barnes et al. nor Komori et al. teaches or suggests the present invention as recited in claims 67-69, 82-83, 86-87. Barnes et al. describes a formulation with a majority DNA polymerase component lacking 3'-5' exonuclease activity (e.g., Taq DNA polymerase) and a minority DNA polymerase component exhibiting 3'-5' exonuclease activity (e.g., wild type Pfu DNA polymerase). Komori et al. describes two specific D405 Pfu mutants (i.e., D405A and D405E) that have reduced polymerase activity. Neither Barnes et al. nor Komori et al. teaches or suggests the claimed invention, that is, an enzyme mixture a first enzyme and a second enzyme, wherein said first enzyme is a DNA polymerase, said second enzyme is a mutant Archaeal DNA polymerase comprising a 3'-5' exonuclease activity and a reduced DNA polymerization activity, wherein when said mutant Archaeal DNA polymerase is a mutant Pfu DNA polymerase, the mutant Pfu DNA polymerase contains a mutation at an amino acid position selected from the group consisting of Y410, T542, D543, K593, Y595, Y385, G387, and G388.

Second, there is no suggestion or motivation to combine the prior art references. In order to establish a prima facie case of obviousness, there must be some reason, suggestion, or motivation from the prior art as a whole that indicates that the person of ordinary skill would have combined or modified the references. The Federal Circuit has stated:

> "[O]bviousness cannot be established by combining the teachings of the prior art to produce the claimed invention, absent some teaching, suggestion or incentive supporting the combination."[2]

As stated above, Barnes et al. describes a formulation with a majority DNA polymerase component lacking 3'-5' exonuclease activity (e.g., Taq DNA polymerase) and a minority DNA polymerase component exhibiting 3'-5' exonuclease activity (e.g., **wild type Pfu DNA polymerase**). Barnes et al. does not teach or suggest a mutant DNA polymerase should be used as the minority DNA polymerase component in the formulation, let alone a mutant Archaeal DNA polymerase comprising a 3'-5' exonuclease activity and a reduced DNA polymerization activity, wherein when said mutant Archaeal DNA polymerase comprising a 3'-5' exonuclease activity and a reduced DNA polymerization activity, the mutant polymerase contains a mutation at an amino acid position selected from the group consisting of Y410, T542, D543, K593, Y595, Y385, G387, and G388.

Komori et al. studies the structure-function relationship of Pfu DNA polymerase, i.e., what mutations affect or abolish the DNA polymerase and exonuclease activities of Pfu:

> "To expand our knowledge of the structure-function relationships fo the family B DNA polymerases, and especially to understand the structural relationship between the DNA polymerizing and 3'-5' exonucleolytic activities in the polymerase protein, we prepared several mutant proteins of Pol BI from *Pfuriosus* by a unidirectional deletion strategy and site-specific mutagenesis, and analyzed their activities." (Page 41, the right column).

> "In conclusion, our mutational analysis further supports the idea that the polymerase and exonuclease domains in the family B DNA polymerases are functionally interdependent. More detailed analyses will be necessary to understand

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[2] *In re Geiger*, 815 F.2d 686, 688, 2 U.S.P.Q.2d 1279, 1278 (Fed. Cir. 1987)

the molecular mechanism of the functional interaction between the two activities in the DNA polymerases." (Page 47, last paragraph).

Komori et al. describes two specific **D405** Pfu mutants (i.e., D405A and D405E) that have reduced polymerase activity. Komori et al. does not teach or suggests that these two mutants can be used with another DNA polymerase in the way claimed in the present invention. **In fact, Komori et al. does not teach that these two Pfu mutants can have any utilities at all.** Even without the present amendments for claims 67-69, 82-83 and 86-87, that is, even if the claims still recite the D405 mutation, one skilled in the art, based on the teachings of Komori et al. and absent of the teachings of Applicants' present invention, would likely avoid mutating the D405 residue of Pfu DNA polymerase to preserve its DNA polymerase and exonuclease activity.

Therefore, nothing in the two references teaches or suggests to combine or modify the references to reach to the present invention, that is, an enzyme mixture comprising a first enzyme and a second enzyme, wherein said first enzyme is a DNA polymerase, said second enzyme is a mutant Archaeal DNA polymerase comprising a 3'-5' exonuclease activity and a reduced DNA polymerization activity, wherein when said mutant Archaeal DNA polymerase comprising a 3'-5' exonuclease activity and a reduced DNA polymerization activity is a mutant Pfu DNA polymerase, the mutant Pfu DNA polymerase contains a mutation at an amino acid position selected from the group consisting of Y410, T542, D543, K593, Y595, Y385, G387, and G388. Because the prior art references fail to provide any suggestion or incentive to combine or modify the references, the Office Action fails to establish a prima facie case of obviousness.

Third, even when the prior art references are combined, they do not result in the present invention as claimed. The amended 67-69, 82-83, 86-87, are drawn to an enzyme mixture comprising a first enzyme and a second enzyme, wherein said first enzyme is a DNA polymerase, said second enzyme is a mutant Archaeal DNA polymerase comprising a 3'-5' exonuclease activity and a reduced DNA polymerization activity, wherein when said mutant Archaeal DNA polymerase comprising a 3'-5' exonuclease activity and a reduced DNA polymerization activity is a mutant Pfu DNA polymerase, the mutant Pfu DNA polymerase contains a mutation at an amino acid position selected from the group consisting of Y410, T542, D543, K593, Y595, Y385, G387, and G388. The combination of Barnes et al. and Komori et al.

still does not teach or suggest an enzyme mixture comprising a first enzyme and a mutant Archaeal DNA polymerase comprising a 3'-5' exonuclease activity and a reduced DNA polymerization activity, wherein when said mutant Archaeal DNA polymerase comprising a 3'-5' exonuclease activity and a reduced DNA polymerization activity is a mutant Pfu DNA polymerase, the mutant Pfu DNA polymerase contains a mutation at an amino acid position selected from the group consisting of Y410, T542, D543, K593, Y595, Y385, G387, and G388.

In view of all of the above, Applicants submit that claims 64-69, 75, 82-83, and 85-87 are not obvious over Barnes et al. and Komori et al. Examiner Hutson agreed during the June 21 interview that such amendment would obviate the 103 rejections over claims 64-69, 75, 82-83, and 85-87.

In view of all of the above, Applicants submit that none of the pending claims are obvious over Barnes et al. and Komori et al., Applicants respectively request the 103(a) rejections over these claims be withdrawn.

Obviousness-type Double Patenting

Claims 64-87 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-3, 6, 9-14, 18, 20-22 and 36-51 of copending Application No. 10/035,091. The Examiner states that although the conflicting claims are not identical, they are not patentably distinct from each other.

While not necessarily acquiescing to the rejection, Applicants submit that they will submit a terminal disclaimer to disclaim any portion of a patent issuing from the present application which would extend beyond the term of a patent issuing from the 10/035,091 application, upon notification of allowable claims in the present application.

CONCLUSION

Applicants submit that in view of the foregoing amendments and remarks, all issues relevant to patentability raised in the Office Action have been addressed. Applicants respectfully request the withdrawal of rejections over the pending claims.

Applicant submits that all claims, i.e., claims 64-94, are allowable as written and respectfully request early favorable action by the Examiner. If the Examiner believes that a telephone conversation with Applicant's attorney/agent would expedite prosecution of this application, the Examiner is cordially invited to call the undersigned attorney/agent of record.

Respectfully submitted,

Date: December 1, 2004

Name: Kathleen M. Williams
Registration No.: 34,380
Customer No.: 27495
Palmer & Dodge LLP
111 Huntington Avenue
Boston, MA 02199-7613
Tel.: (617) 239-0100

Genes Genet. Syst. (1998) 73, p. 323–336

Exhibit A

REVIEW

The euryarchaeotes, a subdomain of Archaea, survive on a single DNA polymerase: Fact or farce?

Yoshizumi Ishino* and Isaac K. O. Cann

Department of Molecular Biology, Biomolecular Engineering Research Institute, 6-2-3 Furuedai, Suita, Osaka 565-0874, Japan

Archaea is now recognized as the third domain of life. Since their discovery, much effort has been directed towards understanding the molecular biology and biochemistry of Archaea. The objective is to comprehend the complete structure and the depth of the phylogenetic tree of life. DNA replication is one of the most important events in living organisms and DNA polymerase is the key enzyme in the molecular machinery which drives the process. All archaeal DNA polymerases were thought to belong to family B. This was because all of the products of *pol* genes that had been cloned showed amino acid sequence similarities to those of this family, which includes three eukaryal DNA replicases and *Escherichia coli* DNA polymerase II. Recently, we found a new heterodimeric DNA polymerase from the hyperthermophilic archaeon, *Pyrococcus furiosus*. The genes coding for the subunits of this DNA polymerase are conserved in the euryarchaeotes whose genomes have been completely sequenced. The biochemical characteristics of the novel DNA polymerase family suggest that its members play an important role in DNA replication within euryarchaeal cells. We review here our current knowledge on DNA polymerases in Archaea with emphasis on the novel DNA polymerase discovered in Euryarchaeota.

INTRODUCTION

For many decades living organisms were thought to cluster into two groups which biologists referred to as prokaryotes and eukaryotes. The prokaryotes were represented by cells without a nucleus, whereas the eukaryotes comprised organisms with nucleated cells which included both unicellular (algae and protozoa) and multicellular (animals, fungi, and plants) organisms. This dogma was radically challenged by Woese and Fox (1977) when they reported the existence of a group of organisms entirely different from bacteria and eukaryotes. At the time of their discovery, the new group of organisms were named archaebacteria but with the proposal that free-living organisms fall into three domains, based on ribosomal RNA comparisons, they were renamed Archaea (Woese et al., 1990). Life on our planet, therefore, comprises Archaea and Bacteria which are prokaryotic in cellular ultrastucture, and Eukarya, which includes all eukaryotes.

The Archaea, despite being prokaryotes, are evolutionarily distinct from the Bacteria and rather appear to share a common ancestor with the Eukarya. Archaea is currently divided into three subdomains (Barns et al., 1996). These are Crenarchaeota, Euryarchaeota, and Korarchaeota (Fig. 1). The organisms falling under Korarchaeota represent a group of as yet to be cultured hyperthermophiles. On the archaeal phylogenetic tree based on 16S rRNA, the korarchaeotes occupy a position very close to the ancestor of this domain. Aside from this information, very little is known about this subdomain. In contrast, many organisms from Euryarchaeota and Crenarchaeota have been isolated and their characteristics well studied. The crenarchaeotes were thought to comprise only of hyperthermophiles growing at temperatures above 80°C (Delong, 1998). One member, *Pyrolobus fumarii*, grows at 113°C (Blochl et al., 1997). There is no other known organism which grows above this temperature. The isolation and characterization of *Cenarchaeum symbiosum*, a psychrophilic (cold-loving) crenarchaoete which grows at 10°C (Preston et al., 1996), however, suggests that the crenarchaeotes thrive within a wide range of temperature. There is more diversity in Euryarchaeota. Members of this subdomain include the extreme halo-

* Corresponding author.

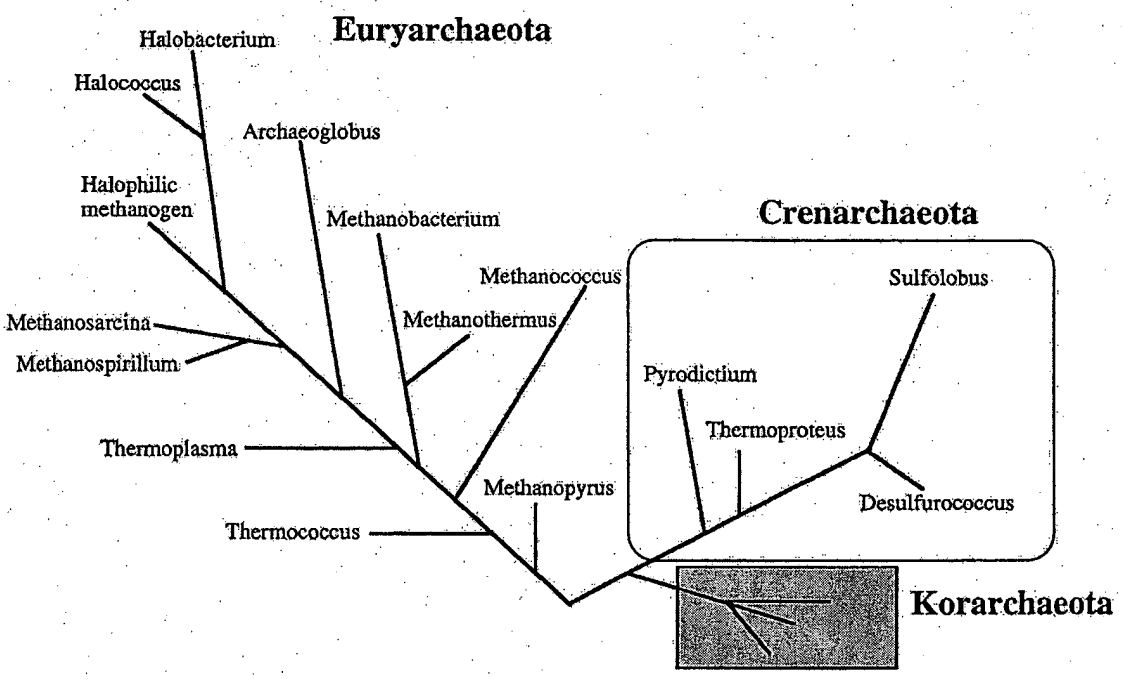

Fig. 1. A phylogenetic tree of Archaea. A phylogenetic tree based on small subunit ribosomal RNA sequence. Archaea is divided into three subdomains, Euryarchaeota, Crenarchaeota, and Korarchaeota.

philes (salt-loving), methanogens (methane-producing), and a group of non-methanogenic, non-halophilic hyperthermophiles including the genera *Thermococcus* and *Pyrococcus*.

The most striking observation from the study of Archaeal molecular biology is the presence of homologs of eukaryotic information processing systems (DNA replication, translation, and transcription) in these organisms. Despite this knowledge, DNA replication in Archaea remains a puzzle due to a lack of identification of essential components as described in very recent review articles (Edgell and Doolittle, 1997; Bernander, 1998). DNA polymerase is one of the most important parts of the molecular machinery in the DNA replication. It is well known that in Bacteria and Eukarya multiple DNA polymerases are involved in DNA metabolic processes (Kornberg and Baker, 1992). The history of the search for archaeal DNA polymerases can be followed by the review article (Forterre et al., 1994). However, our knowledge on the DNA polymerases and DNA replication in Archaea at that time was so fragmentary. The complete genome sequence of the hyperthermophilic euryarchaeote, *Methanococcus jannaschii* (Bult et al., 1996), complicated the issue by suggesting that this organism depends on a single DNA polymerase for its DNA metabolic functions (Edgell and Doolittle, 1996; Gray, 1996; Morell, 1996). Three complete genome reports of other members of this subdomain (Kawarabayasi et al., 1998; Klenk et al., 1997; Smith et al., 1997) seem to substantiate this puzzling observation.

Our research is concentrated on unravelling the mechanism involved in DNA replication in Archaea. Results obtained from our laboratory with archaeal strains show that, indeed, similar to other free-living organisms members of this domain contain multiple DNA polymerases. In this review, we attempt to summarize the recent findings on archaeal DNA polymerases, especially on the discovery of a novel DNA polymerase in *Pyrococcus furiosus*. The DNA polymerase is highly conserved in Euryarchaeota. The current understanding of DNA replication across the three domains of life is also briefly discussed as a prelude to this review.

DNA REPLICATION

DNA replication and repair ensure the maintenance of the integrity of the genome. This is essential for the accurate transfer of genetic information from parent to offspring. However, to confer selective advantage to the progeny, the replication machinery may allow a certain level of mutation in the genome. The process, therefore, plays a central role in the evolution of every species.

The fundamental nature of the DNA replication process is underscored by the conservation of the function of individual proteins in both Bacteria and Eukarya (Stillman, 1994). In summary, the DNA replication process involves 1) recognition of an origin of replication by the origin recognition proteins, 2) melting and unwinding of the duplex parental DNA by a replicative DNA helicase in cooperation with a single-stranded DNA-binding protein,

Table 1. Replication proteins of the three domains of life

Function	Bacterial (*E. cole*)	Eukaryal (yeast/human)	Archaeal
Origin recognition	DnaA	Origin recognition complex (ORC) proteins 1–6	ORC1-like (plasmid-encoded) ORC1-like (*P. furiosus*)
Single-strand DNA-binding	SSB	Replication protein A (RPA; 3 subunits)	archaeal SSB (RPA)
Synthesis of primer	DnaG	DNA polymerase α	α-like DNA polymerases (*H. halobium*)
Helicase	DnaB (5'–3' helicase) PriA (3'–5' helicase)	Dna2 (3'–5' helicase)	archaeal Dna 2
Clamp-loading	γ complex (γδδ'χψ)	Replication factor-C (RFC) 5 subunits (RFC 1-5)	archaeal RFC 1 and 2
Processivity factor	Pol β (DnaN)	Proliferating cell nuclear antigen (PCNA)	archeal PCNA
Synthesis of leading and lagging strand	DNA polymerase III core (αθε), (family C DNA polymerase)	DNA α/ε/δ (Family B DNA polymerase)	Family B DNA polymerase New DNA polymerase family
Ligation of strands on lagging strand	DNA ligase (NAD-dependent)	DNA ligase (ATP-dependent)	DNA ligase (ATP-dependent)
Removal of primers	DNA polymerase I (Family A DNA polymerase) Ribonuclease H	FEN1/RAD2 (*S. pombe*) Ribonuclease H	archael FEN1/Rad2 Ribonuclease H

3) synthesis of a RNA/DNA primer for the leading strand and for each Okazaki fragment on the lagging strand by a primase, 4) the clamp (brace)-loader recognizes the primer/template and loads the sliding clamp which forms a ring around the duplex DNA behind the primer/template junction, 5) the polymerase is loaded onto the DNA, and 6) elongation ensues if all four dNTPs are available. Any experiment analyzing the molecular mechanism of DNA replication in Archaea has not been reported. However many homologs of the proteins working on the eukaryotic replication have been found by the total genome sequences of several archaeal strains. We compared the archaeal homologs to the proteins involved in DNA replication in the two other domains. As shown in Table1, the majority of the protein involved in archaeal chromosome replication are of eukaryal type.

DNA POLYMERASE

Since the establishment of the gene cloning techniques, many genes encoding DNA polymerase have been cloned and sequenced. A proposal to classify DNA polymerases into Family A, B, C, and X on the basis of their amino acid sequences was made (Ito and Braithwaite, 1991). These familes are represented by *Escherichia coli* DNA polymerase I (Family A), DNA polymerase II (Family B), DNA polymerase III α-subunit (Family C), and others such as DNA polymerase β and terminal transferase (Family X).

Most of the biochemical properties of the DNA polymerases in the same family are similar. In Bacteria and Eukarya, several types of DNA polymerases have been isolated and characterized (Fig. 2). *E. coli* DNA polymerases are the most studied enzymes in Bacteria. Pol I and Pol II are the single polypeptide enzymes. Pol III is the DNA replicase of this organism and is a multi-subunit enzyme (10 different subunits). On the otherhand, in Eukarya five DNA polymerases (α, β, δ, ε, and γ) have been characterized in detail. In addition, Pol ζ and η were recently characterized from yeast (Nelson et al., 1996; Sugino, 1995). Pol α, δ, and ε are the DNA replicases and have multisubunit structures. The catalytic subunits of these Pols for DNA polymerizing activity belong to Family B.

In the eukaryotic DNA replication, DNA polymerase α forms a complex with DNA primase to synthesize RNA/DNA primers for initiation of leading strand synthesis and for each Okazaki fragment during lagging strand replication. According to biochemical studies using plasmids containing the Simian Virus 40 origin of replication, it appears that DNA polymerase δ replicates the leading strand and also completes the lagging strand in eukaryal cells. There is therefore a DNA polymerase switch from the DNA polymerase α/primase complex to DNA polymerase δ. On the contrary, in *E. coli* a primase (DnaG)

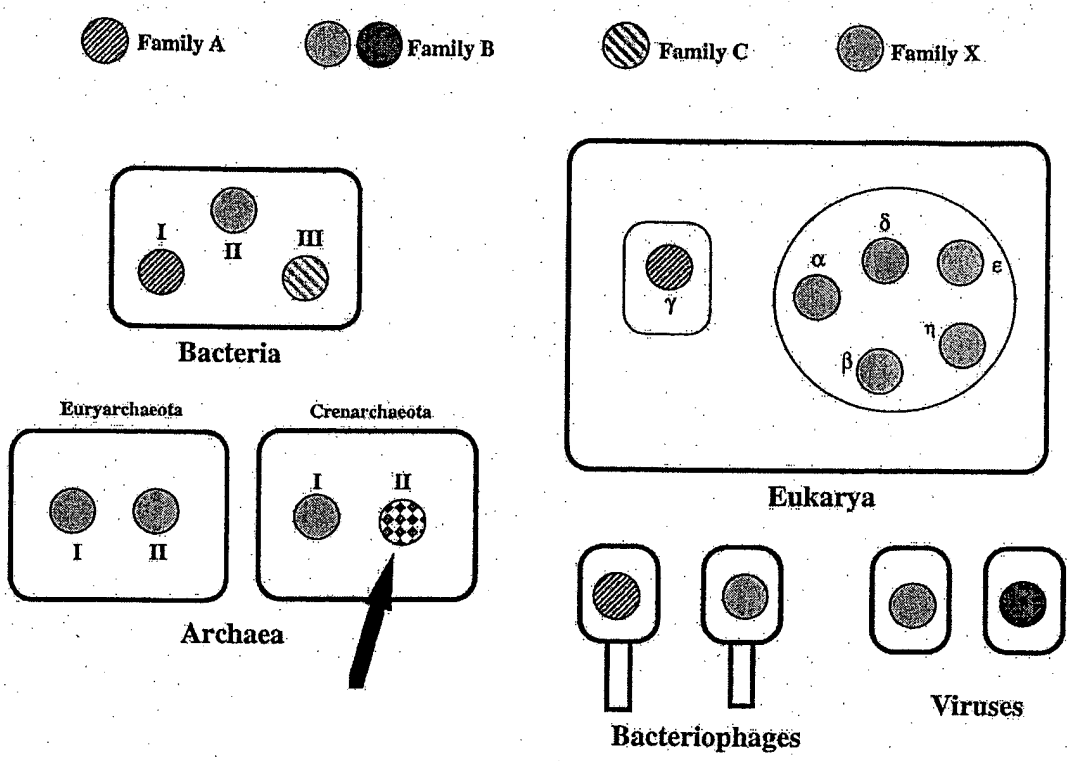

Fig. 2. Distribution of DNA polymerases from family A, B, C, and X among Bacteria, Eukarya, and Archaea. DNA polymerases from the same family are represented by the same pattern.

synthesizes the initial RNA primer which is then elongated by DNA polymerase III core enzymes. The same apparatus synthesizes the lagging strand in a coordinated fashion. Pol δ and ε in Eukarya contain separate domains for DNA polymerizing and 3' • •5' exonucleolytic activities in the same polypeptide. In contrast, in *E. coli* and other gram-negative bacteria the subunit for polymerase activity differs from that for exonuclease activity (α and ε subunit of *E. coli* Pol III). The exonuclease activity is essential for the maintenance of the accurate DNA synthesis.

The molecular mechanism of DNA replication is one of the most interesting subjects in molecular biology, and therefore many review articles have been published. For a thorough account of our current knowledge of DNA replication in eukaryotes and prokaryotes, refer to the following reviews, Stillman (1994), Kelman and O'Donnell (1995), Baker and Bell (1998), Waga and Stillman (1998).

ARCHAEAL FAMILY B DNA POLYMERASES

The study on the archaeal DNA polymerases started by using some halophilic archaea as the subject. Aphidicolin, a tetracyclic diterpene tetraol, which is a specific inhibitor of DNA polymerase α from eukaryal cells (Huberman, 1981), was found to inhibit the growth of *Halobacterium halobium* (Forterre et al, 1984; Schinzel and Burger, 1984) and some by preferentially affecting DNA synthesis. Therefore, it was hypothesized that the DNA replicase of Archaea was similar to that of Eukarya. Subsequently, α-like DNA polymerases were purified from *H. halobium* (Nakayama and Kohiyama, 1985), *Methanococcus vannielii* (Zabel et al., 1985), and *Sulfolobus solfataricus* (Rossi et al., 1986). The biochemical properties of these enzymes were similar to the eukaryotic DNA polymerase α. However, there also have been reports describing the aphidicolin-resistant DNA polymerase activities in *Sulfolobus acidocaldarius* (Klimczak et al., 1985), *Methanobacterium thermoautotrophicum* (Klimczak et al., 1986), *H. halobium* (Nakayama and Kohiyama, 1985), and *Thermoplasma acidophilum* (Hamal et al., 1990). After a while, DNA polymerase genes have been cloned from *S. solfataricus* (Pisani et al., 1992), *Thermococcus litoralis* (Perler et al., 1992), *P. furiosus* (Uemori et al., 1993), *M. voltae* (Konisky, 1994), and *Cenarchaeum symbiosum* (Schleper et al., 1997). The deduced amino acid sequences of all the genes contained the signatures of family B DNA polymerases.

In 1994, two different genes were cloned from *S. solfataricus* P2 cell, both of which seemed to encode family B DNA polymerase (Prangishvili and Klenk, 1994). In 1995, two different family B DNA polymerase genes were cloned and expressed in *E. coli*. Both products actually exhibited DNA polymerase and exonuclease activities

(Uemori et al., 1995). In addition, in the course of sequencing the genome of *S. solfataricus* P2, a gene reputed to code for a third family B DNA polymerase was identified (Edgell et al., 1997), even though the amino acid sequence similarity of this protein to that of other family B members is not strong and there is still skepticism as to whether the gene product has DNA polymerase activity. These are significant findings because they suggest that Archaea and Eukarya, which has three family B DNA polymerases (α, δ, and ε) in the nucleus for DNA replication, share a similar molecular mechanism of DNA replication. *Pyrobaculum aerophilum* has ORFs coding for proteins with similar amino acid sequences to *P. occultum* Pol I and Pol II (Fitz-Gibbon et al., 1997), and very recently, we cloned two genes for family B DNA polymerases from *Aeropyrum pernix* (Cann et al., unpublished), an obligate aerobic hyperthermophilic crenarchaeote (Sako et al., 1996). All these organisms described as having two or three family B DNA polymerases belong to Crenarchaeota. How about Euryarchaeota? We attempted to clone a second gene for a family B DNA polymerase from *P. furiosus*, however, did not succeeded as described below. When the total genome sequence of *M. jannaschii* was published, startling observation on the lack of some important sequences were reported (Bult et al., 1996). One of these inconsistencies was about the DNA polymerase. Only one family B DNA polymerase was found in the whole genome as described above, and no more sequences likely to encode DNA polymerase were found. It would be very surprising if the life of this archaeon was dominated by only a single DNA polymerase. Recent genome sequence projects let us search for the genes for family B DNA polymerases. All strains, *M. jannaschii*, *A. fulgidus*, *M. thermoautotrophicum*, and *P. horikoshii* possess only one family B DNA polymerase gene as well.

The archaeal family B DNA polymerases are overall similar in amino acid sequence, however, they can be divided into two groups. The euryarchaeal family B DNA polymerases are very similar to one of the crenarchaeal family B DNA polymerases, whereas the other is found only in the crenarchaeotes (Fig. 3). From our analyses, *Pfu* Pol I, *Poc* Pol II, and *Ape* Pol II (group 2 in Fig. 3) are sensitive to Aphidicolin at a concentration of less than 2 mM. At a similar aphidicolin concentration, *Poc* Pol I and *Ape* Pol I, and *Soh*Pol I (group 1 in Fig. 3) were resistant (the resistance of Pol I from *Sulfurisphaera ohwakuensis* is a personal communication from N. Kurosawa, Soka Univ.). We hypothesized that the family B DNA polymerase homolog which is common to both subdomains is aphidicholin-sentitive. In contrast, the homolog found only in crenarchaeotes is aphidicolin-resistant. An exception is the protein from *S. solfataricus*, which is sensitive to aphidicolin even though its sequence is more similar to the resistant group (Taguchi and Ishino, unpublished). The difference between the two family B DNA polymerases in terms of the biological roles in the crenarchaeal cells have to be investigated.

Archaeal family B DNA polymerases, especially those from euryarchaeota, often contain inteins. Inteins are intervening sequences that splice as proteins and not as mRNAs (Cooper and Stevens, 1993; Perler et al., 1994). After the production of the precursor protein the intein is excised from the protein and the external protein regions, which are referred to as exteins, are joined together. Archaeal DNA polymerases contain hot spots for insertion of inteins. The regions which are known to contain these inteins are region I, II, and III (originally proposed by Wong et al.,1988, which contain most conserved motifs, motif A, B, and C, proposed by Delarue et al., 1990) in the domain for DNA polymerizing (C-terminal) in Family B DNA polymerases and they are actually important for the formation of the catalytic center of DNA polymerizing activity (Fig. 4). An interesting observation is that, so far, all intein-containing DNA polymerases come from the euryarchaeotes. Two inteins each were found in the precursor proteins from *T. litoralis*, (Perler et al., 1992), *M. jannaschii* (Bult et al., 1996), *Thermococcus fumicolans*, (Cambon and Querellou, 1996), and *Pyrococcus* sp. KOD1 DNA polymerase (Takagi et al., 1997). One intein was found in the precursors from *Pyrococcus* sp. GB-D (Xu et al., 1993), and *P. horikoshii* (personal communication from I. Matsui, Natl. Inst. Biosci. Hum-Tech., Tsukuba).

Archaeal Family B DNA polymerases can practically elongate primers in vitro by themselves, even though they generally have very low processivities, for example, seven dNTP/binding for *T. litoralis* DNA polymerase (Perler et al., 1996). Therefore, some DNA polymerases from hyperthermophilic archaea are commercially available as PCR enzymes. One of the remarkable advantages pertaining to the use of archaeal DNA polymerases for PCR is the high fidelity of synthesis derived from their associated strong 3' • •5' exonuclease activity (Lundberg et al., 1991; Mattila et al., 1991; Takagi et al., 1997).

The three dimensional structure of several nucleotide polymerases using DNA or RNA as a template have been solved as described below. However, any structure of family B DNA polymerase had not been solved until recently. The three dimensional structure of the family B DNA polymerase from *E. coli* bacteriophage RB69 was solved last year and the structure of the catalytic palm domain was found to be basically the same shape as that of other polymerases (Wang et al., 1997). However, the structures of the fingers and thumb domains are unrelated to all other known polymerase structures. Further analyses of the structure-function relationship of the DNA polymerase of this family are still necessary. The family B DNA polymerases from the hyperthermophilic archaea are useful for this purpose, because of the excellent stability of these proteins. Crystal formation of three DNA

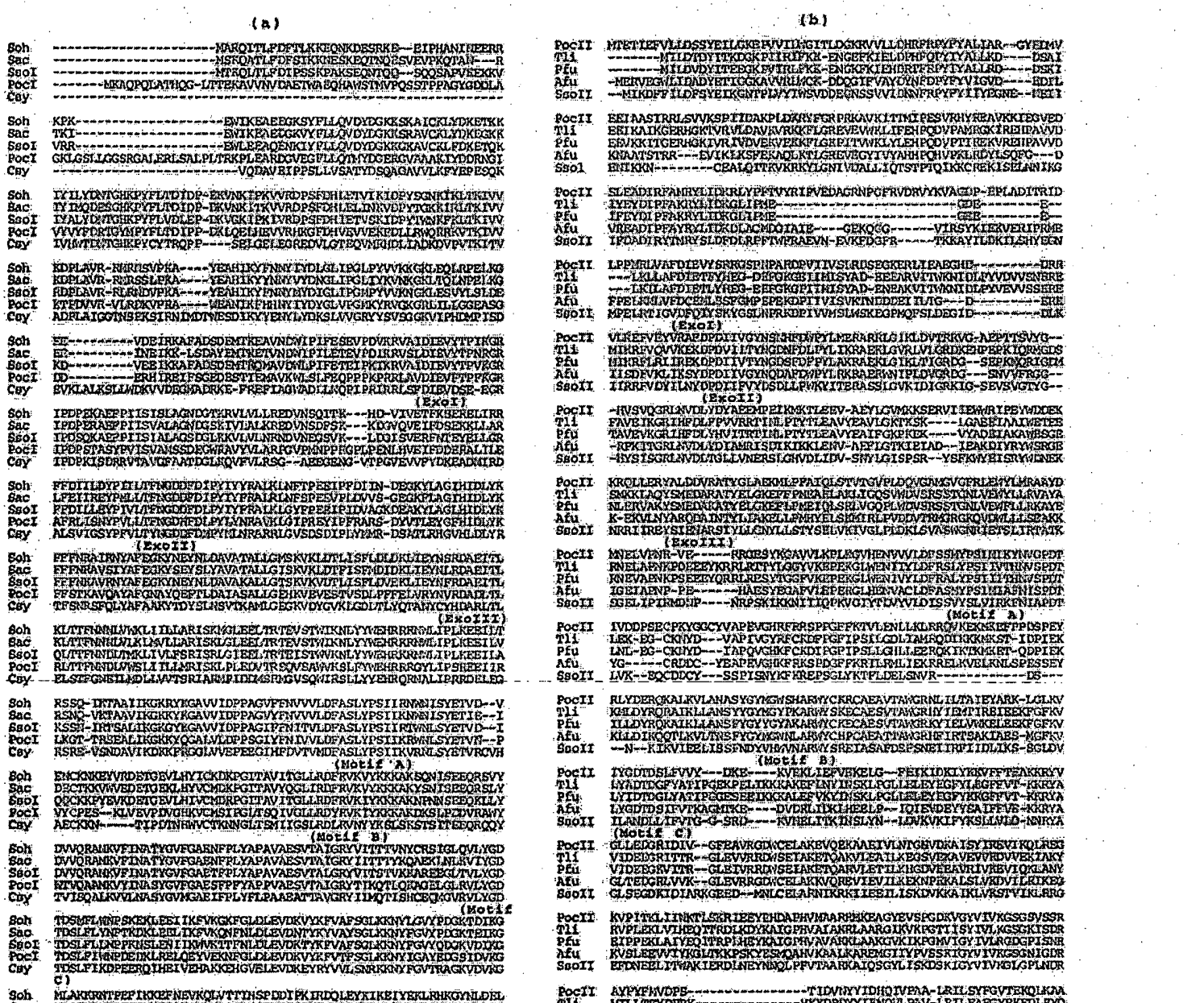

Fig. 3. Amino acid sequence alignment of archaeal family B DNA polymerase homologs found in a) crenarchaeota and b) both crenarchaeota and euryarchaeota. The suffix (I) and (II) are used to indicate the crenarchaeotes which seem to possess two family B DNA polymerases. The euryarchaeotes lack this suffix because of the presence of only one homolog. The proteins and their accession numbers are as follows: SohI (*Sulfurisphaera ohwakuensis* Pol I, AB008894), SacI (*S. acidocaldarius* Pol I, U33846), SsoI (*S. solfataricus* Pol I, U92875), PocI (*P. occultum* Pol I, D38573), CsyI (*C. symbiosum* Pol I, AF028831), PocII (*P. occultum* Pol II, D38574), Tli (*Thermococcus litoralis* Pol, M47198), Pfu (*P. furiosus* Pol, D12983), Afu (*A. fulgidus* Pol, AE001070), SsoII (*S. solfataricus* Pol II, X71597). Identical and similar amino acid residues are indicated by red and green letters, respectively.

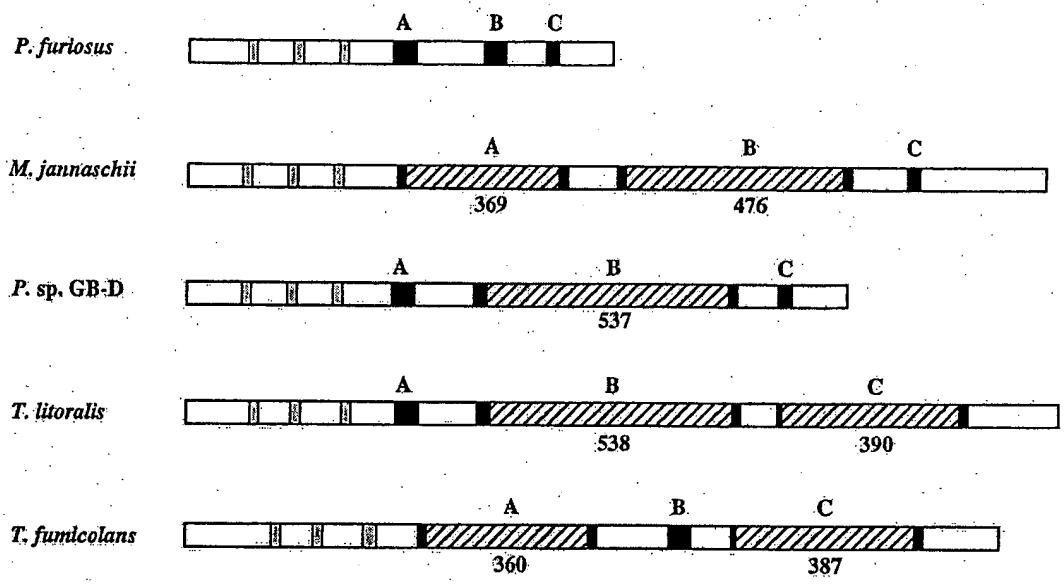

Fig. 4. Schematic comparison of Archaeal family B DNA polymerases having intein in the precursor protein. Black and gray regions represent highly conserved motifs important for polymerase and exonuclease activities, respectively. Inteins are indicated by slanted lines. DNA polymerases from two more *Pyrococcus* (*P.* sp. KOD1 and *P. horikoshii*) have inteins. Insertion positions of KOD1 Pol is the same (motif A and B) as that in *M. jannaschii*, and one intein is inserted in the motif B in Pho Pol same as GB-D Pol.

polymerases and preliminary diffraction analyses have been published (*P. furiosus*, Goldman et al., 1998; *S. solfataricus*, Nastopoulos et al., 1998; *Thermococcus* sp. 9°N-7, Zhou et al., 1998).

EURYARCHAEL HETERODIMERIC DNA POLYMERASE

Background. Despite intensive search through DNA sequencing of PCR products amplified using the degenerate primers, which were designed based on amino acid sequence of conserved motifs in family B DNA polymerases, only one DNA polymerase gene as described above (Uemori et al., 1993) could be isolated from *P. furiosus*. An experiment was, therefore, initiated to determine the number of detectable DNA polymerase activities in the cell extract of *P. furiosus* (Imamura et al., 1995). In the experiment, DNA polymerizing activity was measured by incorporation of [^{3}H]TTP into trichloroacetic acid insoluble products. Three different DNA polymerase activities (I, II, and III) were detected in *P. furiosus* cell extract fractions generated by an anion exchange chromatography (Fig. 5). The activities in fraction I and II were sensitive to aphidicolin which suggested the presence of family B DNA polymerases in these two fractions. The protein eliciting the activity in fraction I was purified and its amino-terminal amino acid sequence was determined by the Edman degradation method (Edman and Begg, 1967). This protein was the family B DNA polymerase (*Pfu* Pol I) previously isolated in *P. furiosus* (Uemori et al., 1993). The DNA polymerase activity in fraction III, unlike those in fractions I and II, was resistant to aphidicolin. In order to purify the protein responsible for this DNA polymerase activity, which was named *Pfu* Pol II, the cell extracts were passed through several purification steps (1, ammonium sulfate; 2, TMAE-anion exchange; 3, phosphocellulose; 4, heparin-sepharose; 5, Sephacryl S-300; 6, 2nd heparin-sepharose). In situ DNA polymerase assay through activity gel analysis (Wernette et al., 1986) suggested that the DNA polymerase activity originated from a protein of molecular mass 130 kDa (Imamura et al., 1995). The activity in fraction II (Fig. 5), which has not been identified, may be a third DNA polymerase in *P. furiosus*. Another possibility is that Pol I might have complexed with some other proteins and eluted at a different place in the chromatography. Further analyses are necessary to solve the question.

The Genes and proteins involved in the formation of euryarchaeal heterodimeric DNA polymerase. A cosmid library containing *P. furiosus* genomic DNA inserts of size ranging from 35 kb – 50 kb was screened for the novel DNA polymerase activity (*Pfu* Pol II) identified in *P. furiosus* cell extracts. Nine out of five hundred independent clones investigated produced heat-stable DNA polymerases. Restriction analysis, using several restriction enzymes, indicated that four positive clones contained the gene for Pol I previously cloned (Uemori et al., 1993), while the remaining five clones had an insert from the same region of *P. furiosus* genomic DNA but different from the gene for Pol I. Within the 8.5-kb *Xba*I fragment containing the genes producing the protein or proteins responsible

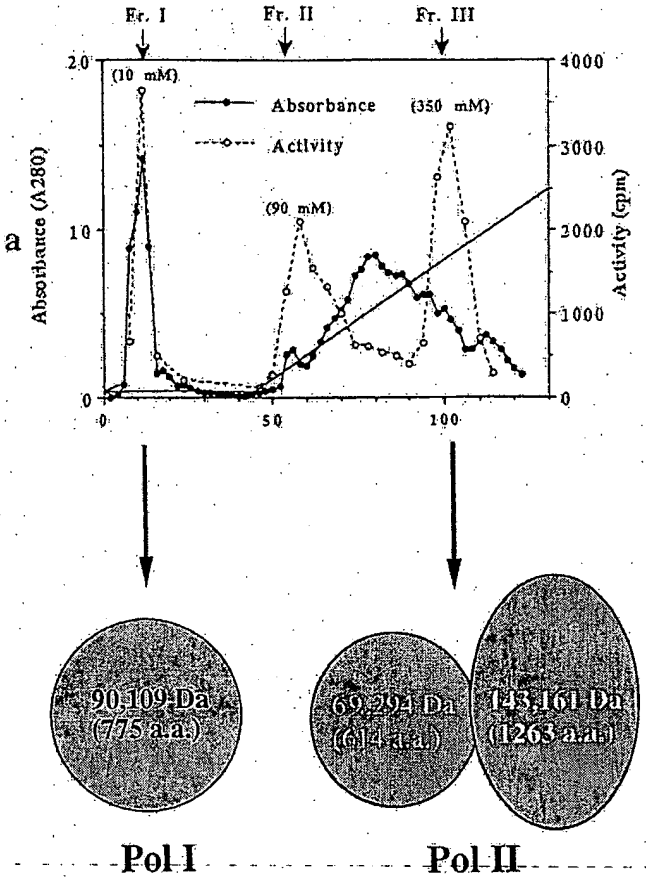

Fig. 5. Chromatographic profiles of an anion-exchange column. The sonicated-crude extract of *P. furiosus* cells was separated by an anion-exchange chromatography. The DNA polymerase activity was measured under standard conditions of [^{3}H]TTP incorporation using calf-thymus activated DNA as template-primers. Pol I and Pol II were purified from fraction I and III, respectively.

for the heat stable DNA polymerase activity, there were five continuous open reading frames (ORFs) transcribed in the same direction (Uemori et al., 1997). Nested deletion analysis showed that the DNA polymerase activity emanated from the second and third genes in the operon. The proteins produced by the second and third ORFs were named DP1 and DP2, respectively (Fig. 6). The estimated molecular mass of DP1 was 67 kDa, while that of DP2 was 139 kDa. The deduced amino-terminal amino acid sequence of DP2 from the nucleotide sequence was completely matched to the experimentaly determined sequence using the purified 130-kDa protein described in Imamura et al. (1995). Even though the 130-kDa protein showed the DNA polymerase activity in an activity gel analysis, which is a very sensitive method for the detection of DNA polymerase activity (Imamura et al., 1995), neither DP1 nor DP2 possesses a distinct DNA polymerase activity individually in an usual in vitro incorporation assay. The activity evolves only in the presence of both proteins (DP1 and DP2) in a reaction mixture (Uemori et al., 1997). Thus it was realized that *Pfu* Pol II comprises two proteins, a small subunit (DP1) and a large subunit (DP2). *Pfu* Pol II possesses a very strong 3' • •5' exonuclease activity. This proof-reading property is also evoked only in the presence of its two components. In addition, Pol II has very efficient primer elongation ability as described below.

It is also interesting that the first and fifth ORFs on the operon including the *pol* genes code for archaeal homologs of Orc1/CDC6 and Rad51/DMC1 of *S. cerevisiae*, respectively as shown in Figure 6 (Uemori et al., 1997). Expecting from the roles of these homologous proteins in yeast cells, the two genes around the genes for *Pfu* Pol II must encode indispensable proteins for DNA replication and recombination in *P. furiosus* cells.

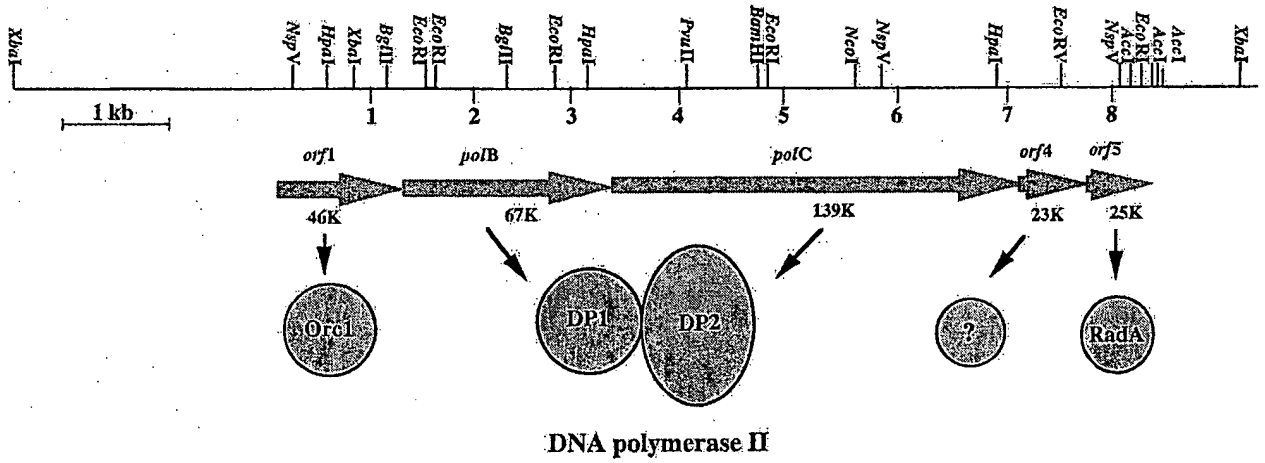

Fig. 6. Restriction map of the DNA fragment containing the gene for *Pfu* Pol II. Open reading frames are indicated by the bold arrows with the deduced molecular weight.

Conservation of Pol II (heterodimeric DNA polymerase) in Euryarchaeota. After the discovery of Pol II from *P. furiosus*, the total genome sequence of *M. jannaschii* was published. Even though, for almost a year, there was a controversy as to whether this organism survives on only one DNA polymerase (family B) as described above, we already knew that it has at least one more DNA polymerase. In the *M. jannaschii* genome, we found two ORFs coding for proteins which are homologous to *Pfu* DP1 (40% identity) and *Pfu* DP2 (60% identity), respectively. The genes for these two ORF were expressed in *E. coli*, and the proteins were confirmed to possess actually DNA polymerase and 3' • •5' exonuclease activities (Ishino et al., 1998). Subsequently, homologs of both DP1 and DP2 were found in the three more euryarchaeal complete genome sequences of *M. thermoautotrophicum*, *A. fulgidus*, and *P. horikoshii* (Cann et al., 1998). In each of these organisms DP1 and DP2 are highly conserved (Table 2, Fig. 7). In addition, using primers based on conserved motifs in DP2, we have demonstrated the presence of a homolog of this protein in *Methanopyrus kandleri* (Cann and Ishino, unpublished), which is the most ancient of all known hyperthermophilic archaea (Fig. 1). Every strain which has, so far, been shown to contain DP1 and DP2 belongs to Euryarchaeota. There is no evidence suggesting the presence or absence of DP1 and DP2 homologs in crenarchaeal cells. However, the conservation of the genes for DP1 and DP2 in euryarchaeotes signifies that these proteins play an important role in these organisms.

The DP2 of *P. horikoshii* contains an intein comprising 179 amino acids in a highly conserved motif, GYAHYFHAAKRRNCDGDED, in DP2 proteins (Fig. 7). In many cases genes coding for inteins are located in indispensable motifs as described above for DNA polymerases. An intein also occurs in a Walker's motif A that is important for NTP binding of replication factor C small subunit of *P. furiosus* (Cann and Ishino, unpublished). The function of the motif in which the intein occurs in *P. horikoshii* DP2 is not known, but it is very close to the putative catalytic residues of Pol II (Cann et al., 1998). Therefore, it may be important for the conformation of the active site. Mutagenesis within the motif is likely to yield some interesting results.

The genes coding for DP1 and DP2 are arranged in tandem in the genus *Pyrococcus* (*P. furiosus*, Uemori et al., 1997; *P. woesei*, Cann and Ishino, unpublished; *P. horikoshii*, Kawarabashi et al., 1998). However, in other genera the genes occur at different regions of the genome as described earlier (Ishino et al., 1998). The difference of these gene organization between forming an operon and locating at separated places may affect the production level of Pol II in the cells.

Biochemistry of euryarchaeal heterodimeric DNA polymerase. The biochemical characteristics of *M. jannaschii* Pol II produced in *E. coli* have been compared with those of *Pfu* Pol II (Ishino et al., 1998). Both *Mja* DP1 and DP2 reacted to a polyclonal antibody against *Pfu* Pol II. Similar to *Pfu* Pol II, *Mja* Pol II possesses an extremely active 3' • •5' exonuclease activity. The DNA polymerase activity of each Pol II is sensitive to N-ethylmaleimide (NEM), but resistant to aphidicolin. Both Pol IIs are more sensitive to ddTTP and salt (KCl) than *Pfu* Pol I. These responses by the euryarchaeal heterodimeric DNA polymerase to the above reagents are different from that of other known DNA polymerases (Kornberg and Baker, 1992).

The subunits of *Pfu* Pol II can complement *Mja* Pol II subunits to yield DNA polymerase activity and vice versa, though an incompatibility to a certain extent between *Pfu* DP1 and *Mja* DP2 was observed (Fig. 8). *Pfu* Pol II is more heat stable than *Mja* Pol II. Incubations at 94°C for 20 min did not affect DNA polymerase activity of *Pfu* Pol II. Indeed, activity rather seemed to improve with longer periods of pre-incubation at this temperature. Significant loss of activity occurred in *Mja* Pol II at incu-

Table 2. Identities of DP1 and DP2 amino acid sequences among four archaeal strains

	identity (%)			
DP1	*P. furiousus*	*A. fulgidus*	*M. jannaschii*	*M. thermoautotrophicum*
P. furiousus	—			
A. fulgidus	38.4 (48.8)	—		
M. jannaschii	32.9 (46.1)	41.4 (53.3)	—	
M. thermoautotrophicum	36.9 (49.0)	42.6 (51.4)	35.8 (46.9)	—
DP2				
P. furiosus	—			
A. fulgidus	52.6	—		
M. jannaschii	54.3	52.7	—	
M. thermoautotrophicum	53.4	50.2	53.1	—

The values in brackets are from the comparison of the carboxyl-terminus beginning from the conserved sequences SD · H · GS.

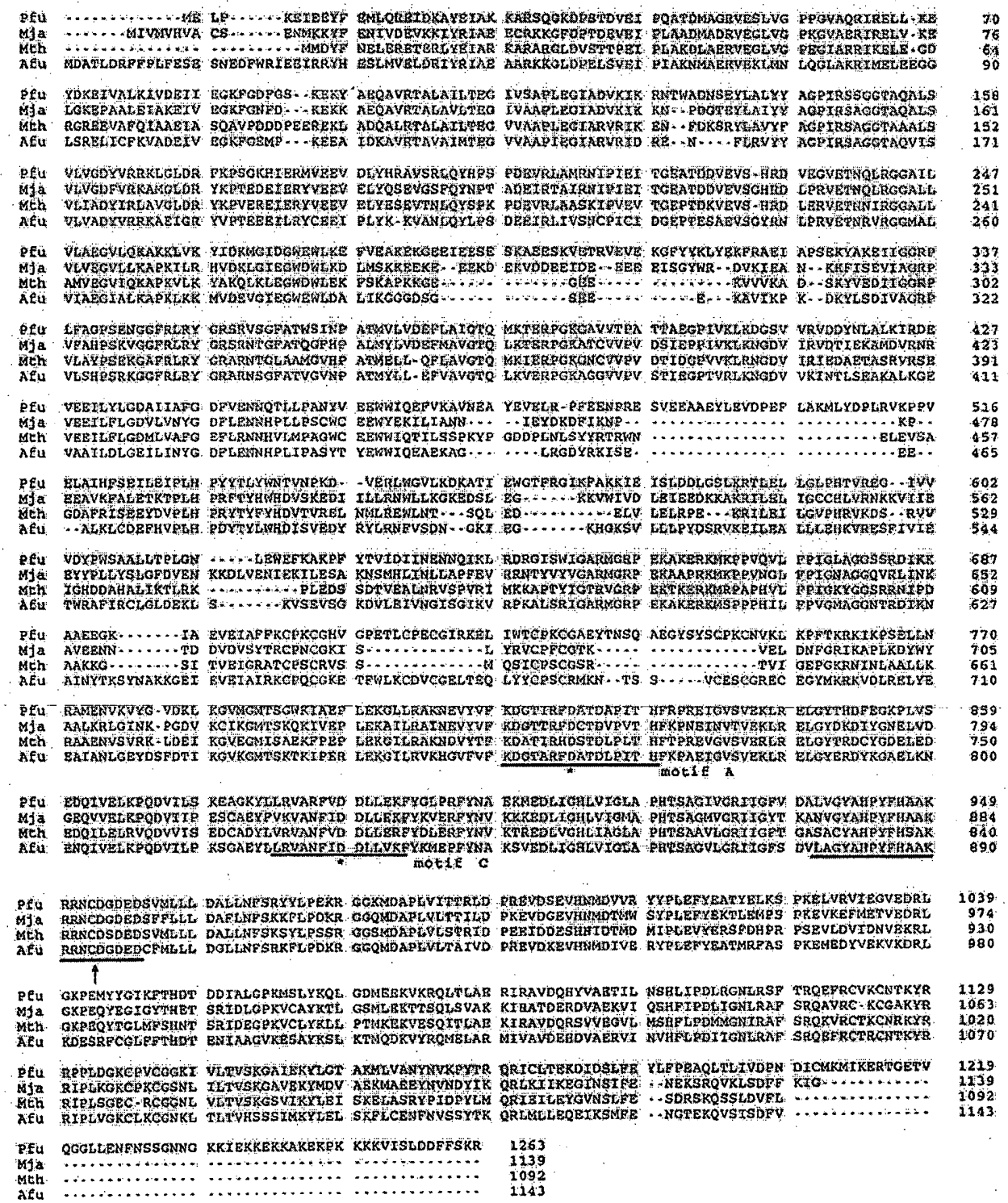

Fig. 7. Amino acid sequence alignment of euryarchaeal DP2 proteins. The DP2s shown are from *P. furiosus* (Pfu), *M. jannaschii* (Mja), *M. thermoautotrophicum* (Mth), and *A. fulgidus* (Afu). Identical and similar amino acid residues are indicated by red and green letters, respectively. The motif A and C were underlined and the asterisks indicate invariant residues probably essential for the catalysis. The intein-insertion site within the DP2 from *P. horikoshii* (the whole sequence is not included in this alignment) is indicated by an arrow.

bations above 65°C. When *Mja* DP1 was complemented with *Pfu* DP2, temperature stability was significantly improved (Ishino et al., unpublished).

From the study of purified Pol I and Pol II of *P. furiosus*, several distinct differences in the chracteristics were found (Uemori et al., 1997). Pol II prefers the single-

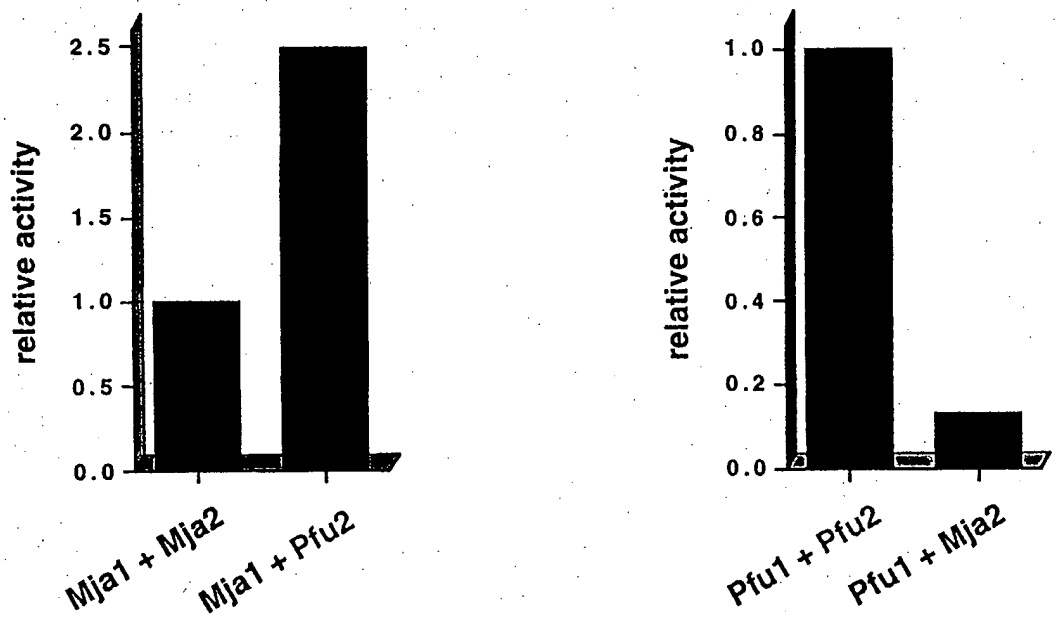

Fig. 8. Inter-subunit complementation of euryarchaeal Pol II. Relative activities of chimeric Pol IIs (*Pfu* DP1 + *Mja* DP2 and *Mja* DP1 + *Pfu* DP2) in comparison to the homotaric Pol IIs are shown. The DNA polymerase activity was measured by [^{3}H]TTP incorporation assay at 70°C for 10 min under standard conditions using calf-thymus activated DNA as template-primers.

Table 3. Comparison of DNA polymerase activities on various template-primers

Template-primer	Relative Activity				
	DP1	DP2	DP1 + DP2	*Pfu* PolI	*Taq*
Calf thymus activated DNA	0.021	0.19	100	100	100
Heat-denatured calf thymus activated DNA			340	87	130
M13 ssDNA-45 mer primer			170	23	90
M13 ssDNA-18 mer (RNA) primer			52	0.49	38
Poly dA-Oligo dT (20 : 1)			94	390	290
Poly A-Oligo dT (20 : 1)			0.085	—	0.063

primed template DNA rather than gapped double-stranded DNA such as DNase I-activated DNA. This is in contrast to *Pfu* Pol I. Moreover, Pol II can utilize RNA primers, whereas Pol I can not (Table 3). The specific activity of Pol II using an activated calf thymus DNA as a template was 191.3 units/nmol while that of Pol I was 88.3 units/nmol. The strong primer elongation ability of Pol II supports the idea that this enzyme is a replicase of the euryarchaeotes.

Comparison of euryarchaeal heterodimeric DNA polymerase with known DNA polymerases. By amino acid sequence comparison of DP1s from four euryarchaeal Pol I, we showed that this protein is a homolog of the small subunit of eukaryal DNA polymerase δ (Cann et al., 1998). This finding further substantiates the eukaryal-archaeal relationship. The amino acid sequence alignment between eukaryal and euryarchaeal homologs indicated several conserved motifs which may play a role in the function of this protein. Amino acid sequence conservation is most distinct at the carboxyl-terminal region of the alignment, while diversity is observed at the amino-terminal region. There are conserved motifs in the central region of the alignment which are unique to the euryarchaeal homologs. These motifs may be important for the interactions either between DP1 and DP2 or between DP1 and other accessory proteins. Our deletion analyses showed that the carboxyl-terminal two-third of the DP1 protein is especially important for its interaction with DP2 to yield DNA polymerase activity of Pol II (Hayashi et al., unpublished). The proteins from *P. furiosus*, *P. horikoshii* and *M. jannaschii* are significantly larger than other euryarchaeal (*M. thermoautotrophicum* and *A. fulgidus*) and eukaryal homologs (Cann et al., 1998).

It is of interest to note that the large subunit of eukaryal

DNA polymerase δ also belongs to family B as well as the archaeal single subunit DNA polymerases, however, *Pfu* DP1 does not interact with *Pfu* Pol I (Cann et al., 1998). This indicates that euryarchaeal DP1s and DP2s are specific partners in the formation of a DNA polymerase. The formation of a complex between DP1 and DP2 in the *P. furiosus* cells have been demonstrated by immunological methods (Cann et al., 1998).

Computer-assisted homology analyses using the World Wide Web (http://www.ncbi.nlm.nih.gov/) retrieval system (National Library of Medicine) and the BLAST algorithm (Altschul et al., 1990) to scan GenBank and other non-redundant databases, did not yield any protein of meaningful homology to the DP2 protein. Conservation of the protein in euryarchaeotes, however, is very high (Table 2). There is more than 50% amino acid conservation among known DP2s. The crystal structures determined for nucleotide polymerases show that these proteins share a common folding pattern that resembles a right hand composed of the fingers, thumb, and palm subdomains (Hansen et al., 1997; Kiefer et al., 1997; Kim et al., 1995; Kohlstaedt et al., 1992; Ollis et al., 1985; Sousa et al., 1993). Within the palm subdomain are two motifs (motif A and C) containing two invariant carboxylates, which are thought to constitute part of the polymerase active site. The amino acid sequences weakly resembling motif A and C were found in DP2s but not in DP1s by visual inspection of four euryarchaeal DP2s (Fig. 7). Therefore, the DP2s have been proposed as the catalytic subunit of the euryarchaeal heterodimeric DNA polymerase, despite showing insignificant DNA polymerase activity in vitro by itself as described above.

In the middle and carboxyl-terminal regions of DP2s, zinc-finger motifs which are likely to be involved in interactions with other proteins, in addition to DNA binding, are conserved. Proliferating cell nuclear antigen (PCNA) is a highly conserved eukaryotic protein that is essential for DNA replication and repair (Johnson and Hubscher, 1997; Kelman, 1997). An euryarchaeal homolog of this protein occurs in all completely sequenced euryarchaeal genomes. At the caxboxyl-terminal region of all known DP2s are two conserved motifs similar to the so-called PIP (PCNA interacting protein)-box (Warbick, 1998). Similar conserved amino acid sequences also occur in the large subunit of euryarchaeal replication factor C (RFC) homologs (data not shown). It is our hypothesis that euryarchaeal DP2s interact with PCNA homologs found in euryarchaeota. Experiments investigating these interactions are now underway in our laboratory. It is also interesting to investigate the phylogenetic relationship between the two DNA polymerases (heterodimeric and family B) found in Archaea.

CONCLUDING REMARKS

Archaea is now paid a lot of attention in molecular biology because their genetic information processing apparatus looks more like that of eukaryotic organisms, even though they look like bacteria. Despite this clue, the basic molecular mechanism of DNA replication in these organisms is not yet understood. Undoubtedly, a full understanding of DNA replication in Archaea requires the identification of all the proteins involved. The discovery of the novel DNA polymerase family, which are probably involved in the DNA replication machinery, will greatly contribute to the understanding of the mechanism, in addition to that it serves as a further confirmation of the archaeal organisms to be truly different from those in Bacteria and Eukarya.

Currently archaeal homologs involved in the eukaryal DNA replication, such as minichromosome maintenance (Mcm) proteins, replication protein A (RPA), RFC, PCNA, 5' • •3' exo/endonuclease (FEN1), and DNA helicase are studied at several laboratories in the world. Orc1/CDC6-like protein will help to identify the replicational origin in the genome very soon. The existence of the proteins similar to the essential factors for eukaryal DNA replication in Archaeal cells let us expect that the archaeal mechanism will contribute as the prototype to the understanding of the mechanism in eukaryal DNA replication that became very much complicated by evolution. We now recognize the archaeal organisms as one of the most exciting and useful experimental materials for the basic molecular biology.

Most of our studies on DNA polymerases from *P. furiosus* cited in this article were carried out in Biotechnology Research Laboratories, Takara Shuzo in collaboration with I. Kato, T. Uemori, and other members.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. J. Mol. Biol. **215**, 403–410.

Baker, T. A., and Bell, S. P. (1998) Polymerases and the replisome: machines within machines. Cell **92**, 295–305.

Barns, S. M., Delwiche, J. D., Palmer, J. D., and Pace, N. R. (1996) Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. Proc. Natl. Acad. Sci. USA **93**, 9188–9193.

Bernander, R. (1998) Archaea and the cell cycle. Mol. Microbiol. **29**, 955–961.

Blochl, E., Rachel, R., Burggraf, S., Hafenbradl, D., Jannasch, H. W., and Stetter, K. O. (1997) *Pyrolobus fumarii*, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113°C. Extremophiles 1, 14–21.

Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, R. D., Sutton, G. G, Blake, J. A., FitzGerald, L. M., Clayton, R. A., Gocayne, J. D., Kerlavage, A. R., Dougherty, B. A., Tomb, J.-F., Adams, M. D., Reich, C. I., Overbeek, R., Kirkness,

E. F., Weinstock, K. G., Merrick, J. M., Glodek, A., Scott, J. L., Geoghagen, N. S. M., Weidman, J. F., Fuhrmann, J. L., Presley, E. A., Nguyen, D., Utterback, T. R., Kelley, J. M., Peterson, J. D., Sadow, P.W., Hanna, M. C., Cotton, M. D., Hurst, M. A., Roberts, K. M., Kaine, B. P., Borodovsky, M., Klenk, H.-P., Fraser, C. M., Smith, H. O., Woese, C. R., and Venter, J. C. (1996) Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science **273**, 1058–1073.

Cambon, M., and Querellou, J. (1996) Gene organization of DNA polymerase of the archaeon *Thermococcus fumicolans*. Direct submission to the Data base. Accession number: Z69882.

Cann, I. K. O., Komori, K., Toh, H., Kanai, S., and Ishino, Y. (1998) A heterodimeric DNA polymerase: evidence that members of euryarchaeota possess a distinct DNA polymerase. Proc. Natl. Acad. Sci. USA **95**, 14250–14255.

Cooper, A. A., and Stevens, T. H. (1993) Protein splicing: excision of intervening sequences at the protein level. Bioessays **15**, 667–74.

Delarue, M., Poch, O., Tordo, N., Moras, D., and Argos, P. (1990) An attempt to unify the structure of polymerases. Protein Eng. **3**, 461–467.

Delong, E. (1998) Archaeal means and extremes. Science **280**, 542–543.

Edgell, D. R., and Doolittle, W. F. (1996) Archaebacterial genomics: the complete genome sequence of *Methanococcus jannaschii*. Bioessays **19**, 1–4.

Edgell, D. R., and Doolittle, W. F. (1997) Archaea and the origin(s) of DNA replication proteins. Cell **89**, 995–998.

Edgell, D. R., Klenk, H-P., and Doolittle, W. F. (1997) Gene duplication in evolution of archaeal family B DNA polymerases. J. Bacteriol. **179**, 2632–2640.

Edman, P., and G. Begg, G. (1967) A protein sequenator. Eur. J. Biochem. **1**, 80–91.

Fitz-Gibbon, S., Choi, A. J., Miller, J. H., Stetter, K. O., Simon, M. I., Swanson, R., and Kim, U.-J. (1997) A fosmid-based genomic map and identification of 474 genes of the hyperthermophilic archaeon *Pyrobaculum aerophilum*. Extremophiles **1**, 36–51.

Forterre, P., Elie, C., and Kohiyama, M. (1984) Aphidicolin inhibits growth and DNA synthesis in halophilic arachaebacteria. J. Bacteriol. **159**, 800–802.

Forterre, P., Bergerat, A., Gadelle, D., Elie, C., Lottspeich, F., Confalonieri, F., Duguet, M., Holmes, M., and Dyall-Smith, M. (1994) Evolution of DNA topoisomerases and DNA polymerases: a perspective from archaea. Syst. Appl. Microbiol. **16**, 746–758.

Goldman, S., Kim, R., Hung, L.-W., Jancarik, J., and Kim, S.-H. (1998) Purification, crystallization and preliminary X-ray crystallographic analysis of *Pyrococcus furiosus* DNA polymerase. Acta Crystallogr. Sect. **D54**, 986–988.

Gray, M. W. (1996) The third form of life. Nature **383**, 299.

Hamal, A., Forterre, P., and Elie, C. (1990) Purification and characterization of a DNA polymerase from the archaebacterium *Thermoplasma acidophilum*. Eur. J. Biochem. **190**, 317–521.

Hansen, J. L., Long, A. M., and Schultz, S. C. (1997) Structure of the RNA-dependent RNA polymerase of poliovirus. Structure **5**, 1109–1122

Huberman, J. A. (1981) New view of the biochemistry of eukaryotic DNA replication revealed by aphidicolin, an unusual inhibitor of DNA polymerase α. Cell **23**, 647–648.

Imamura, M., Uemori, T., Kato, I., and Ishino, Y. (1995) A non-α-like DNA polymerase from the hyperthermophilic *archaeon Pyrococcus furiosus*. Biol. Pharm. Bull. **18**, 1647–1652.

Ishino, Y., Komori, K., Cann, I. K. O., and Koga, Y. (1998) A novel DNA polymerase family found in Archaea. J. Bacteriol. **180**, 2232–2236.

Ito, J., and Braithwaite, D. K. (1991) Compilation, alignment, and phylogenetic relationships of DNA polymerases. Nucleic Acids Res. **19**, 4045–4057.

Johnson, Z. O., and Hubscher, U. (1997) Proliferating cell nuclear antigen: More than a clamp for DNA polymerases. Bioessays **19**, 967–975.

Kawarabayasi, Y., Sawada, M., Horikawa, H., Haikawa, Y., Hino, Y., Yamamoto, S., Sekine, M., Baba, S., Kosugi, H., Hosoyama, A., Nagai, Y., Sakai, M., Ogura, K., Otsuka, R., Nakazawa, H., Takamiya, M., Ohfuku, Y., Funahashi, T., Tanaka, T., Kudoh, Y., Yamazaki, J., Kushida, N., Oguchi, A., Aoki, K., Yoshizawa, T., Nakamura, Y., Robb, F. T., Horikoshi, K., Masuchi, Y., Shizuya, H., and Kikuchi, H. (1998) Complete sequence and gene organization of the genome of a hyper-thermophilic archaebacterium, *Pyrococcus horikoshii* OT3. DNA Res. **5**, 55–76.

Kelman, Z. (1997) PCNA: structure, functions and interactions. Oncogene **14**, 629–640.

Kelman, Z., and O'Donnell, M. (1995) DNA polymerase III holoenzyme: structure and function of a chromosomal replicating machine. Annu. Rev. Biochem. **64**, 171–200.

Kiefer, J. R., Mao, C., Hansen, C. J., Basehore, S. L., Hogrefe, H. H., Braman, J. C., Beese, L. S. (1997) Crystal structure of a thermostable *Bacillus* DNA polymerase I large fragment at 2.1 A resolution. Structure **5**, 95–108.

Kim, Y., Eom, S. H., Wang, J., Lee, D.-S., Suh, S. W., and Steitz, T. A. (1995) Crystal structure of *Thermus aquaticus* DNA polymerase. Nature **376**, 612–616.

Klenk, H. P., Clayton, R. A., Tomb, J., White, O., Nelson, K. E., Ketchum, K. A., Dodson, R. J., Gwinn, M., Hickey, E. K., Peterson, J. D., Richardson, D. L., Kerlavage, A. R., Graham, D. E., Kyrpides, N. C., Fleischmann, R. D., Quackenbush, J., Lee, N. H., Sutton, G. G., Gill, S., Kirkness, E. F., Dougherty, B. A., McKenney, K., Adams, M. D., Loftus, B., Peterson, S., Reich, C. I., McNeil, L. K., Badger, J. H., Glodek, A., Zhou, L., Overbeek, R., Gocayne, J. D., Weidman, J. F., McDonald, L., Utterback, T., Cotton, M. D., Spriggs, T., Artiach, P., Kaine, B. P., Sykes, S. M., Sadow, P. W., D'Andrea, K. P., Bowman, C., Fujii, C., Garland, S. A., Mason, T. M., Olsen, G. J., Fraser, C. M., Smith, H. O., Woese, C. R., and Venter, J. C. (1997) The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*. Nature **390**, 364–370.

Klimczak, L. J., Grummt, F., and Burger, K. J. (1985) Purification and characterization of DNA polymerase from the archaebacterium *Sulfolobus acidcaldarius*. Nucleic Acids Res. **13**, 5269–5282.

Klimczak, L. J., Grummt, F., and Burger, K. J. (1986) Purification and characterization of DNA polymerase from the archaebacterium *Methanobacterium thermoautotrophicum*. Biochemistry **25**, 4850–4855.

Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992) Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science **256**, 1783–1790.

Konisky, J., Paule, S. M., Carinato, M. E., and Kansy, J. W. (1994) The DNA polymerase gene from the methanogenic archaeon *Methanococcus voltae*. J. Bacteriol. **176**, 6402–6403.

Kornberg, A., and Baker, T. A. (1992) DNA Replication, 2nd ed. W. H. Freeman and Co., New York.

Lundberg, K. S., Shoemaker, D. D., Adams, M. W. W., Short, J. M., Sorge, J. A., and Mathur, E. J. (1991) High-fidelity amplification using a thermostable DNA polymerase isolated

from *Pyrococcus furiosus*. Gene **108**, 1–6.
Mattila, P., Korpela, J., Tenkanen, T., and Pitkanen, K. (1991) Fidelity of DNA synthesis by the *Thermococcus litralis* DNA polymerase — an extremely heat stable enzyme with proofreading activity. Nucleic Acids Res. **19**, 4967–4973.
Morell, V. (1996) Life's last domain. Science **273**, 1043–1045.
Nakayama, M., and Kohiyama, M. (1985) An α-like DNA polymerase from *Halobacterium halobium*. Eur. J. Biochem. **152**, 293–297.
Nastopoulos, V., Pisani, F., Savino, C., Federici, L, Rossi, M., and Tsernoglou, D. (1998) Crystallization and preliminary X-ray diffraction studies of DNA polymerase from the thermophilic archaeon *Sulfolobus solfataricus*. Acta Crystallogr. Sect. **D54**, 1002–1004.
Nelson, J. R., Lawrence, C. W., and Hinkle, D. C. (1996) Thymine-thymine dimer bypass by yeast DNA polymerase zeta. Science **272**, 1646–1649.
Ollis, D. L., Brick, P., Hamlin, R., Xuong, N. G., and Steitz, T. A. (1985) Structure of large fragment of *Escherichia coli* DNA polymerase I complexed with dTMP. Nature **313**, 762–766.
Perler, F. B., Davis, E. O., Dean, G. E., Gimble, F. S., Jack, W. E., Neff, N., Noren, C. J., Thorner, J., and Belfort, M. (1994) Protein splicing elements: inteins and exteins — a definition of terms and recommended nomenclature. Nucleic Acids Res. **22**, 1125–1127.
Perler, F. B., Kumar, S., and Kong, H. (1996) Thermostable DNA polymerases. Adv. Protein Chem. **48**, 377–435.
Perler, F. B., Comb, D. G., Jack, W. E., Moran, L. S., Qiang, B., Kucera, R. B., Benner, J, Slatko, B. E., Nwankwo, D. O., Hempstead, S. K., Carlow, C. K. S., and Jannasch, H. (1992) Intervening sequences in an Archaea DNA polymerase gene. Proc. Natl. Acad. Sci. USA. **89**, 5577–5581.
Pisani, F., De Martino, C., and Rossi, M. (1992) A DNA polymerase from the archaeon *Sulfolobus solfataricus* shows sequence similarity to family B DNA polymerases. Nucleic Acids Res. **20**, 2711–2716.
Prangishvili, D., and Klenk, H-P. (1994) The gene for a 74 kDa DNA polymerase from the Archaeon *Sulfolobus solfataricus*. Syst. Appl. Microbiol. **16**, 665–671.
Preston, C. M., Wu, K. Y., Molinski, T. F., and DeLong, E. F. (1996) A psychrophylic crenarchaeon inhabits a marine sponge: *Cenarchaeum symbisum* gen. nov., sp. nov. Proc. Natl. Acad. Sci. USA **93**, 6241–6246.
Rossi, M., Rella, R., Pensa, M., Bartolucci, S., De Rosa, M., Gambacorta, A., Raia, C. A., and Dell'Aversano Orabona, N. (1986) Structure and properties of a thermophilic and thermostable DNA polymerase isolated from *Sulfolobus solfataricus*. Syst. Appl. Microbiol. **7**, 337–341.
Sako, Y., Nomura, N., Uchida, A., Ishida, Y., Morii, H., Koga, Y., Hoaki, T., and Maruyama, T. (1996) *Aeropyrum pernix* gen. nov., sp. nov., a novel aerobic hyperthermophilic archaeon growing at temperatures up to 100°C. Int. J. Syst. Bacteriol. **46**, 1070–1077.
Schinzel, R., and Burger, K. J. (1984) Sensitivity of halobacteria to aphidicolin, an inhibitor of eukaryotic α-type DNA polymerases. FEMS Microbiol. Lett. **25**, 187–190.
Schleper, C., Swanson, R. V., Mathur, E. J., and Delong, E. F. (1997) Characterization of a DNA polymerase from the uncultivated psychrophilic archaeon *Cenarchaeum symbiosum*. J. Bacteriol. **179**, 7803–7811.
Smith, D. R., Doucette-Stamm, L. A., Deloughery, C., Lee, H.-M., Dubois, J., Aldredge, T., Bashirzadeh, R., Blakely, D., Cook, R., Gilbert, K., Harrison, D., Hoang, L., Keagle, P., Lumm, W., Pothier, B., Qiu, D., Spadafora, R., Vicare, R., Wang, Y., Wierzbowski, J., Gibson, R., Jiwani, N., Caruso, A., Bush, D., Safer, H., Patwell, D., Prabhakar, S., McDougall, S., Shimer, G., Goyal, A., Pietrovski, S., Church, G.M., Daniels, C. J., Mao, J.-i., Rice, P., Nolling, J., and Reeve, J. N. (1997). Complete genome sequence of *Methanobacterium thermoautotrophicum* ΔH: Functional analysis and comparative genomics. J. Bacteriol. **179**, 7135–7155.
Sousa, R., Chung, Y. J., Rose, J. P., and Wang, B.-C. (1993) Crystal structure of bacteriophage T7 RNA polymerase at 3.3 A resolution. Nature **364**, 593–599
Stillman, B. (1994) Smart machines at the DNA replication fork. Cell **78**, 725–728.
Sugino, A. (1995) Yeast DNA polymerases and their role at the replication fork. Trends Biochem. Sci. **20**, 319–323.
Takagi, M., Nishioka, M., Kakihara, H., Kitabayashi, M., Inoue, H., Kawakami, B., Oka, M, and Imanaka, T. (1997) Characterization of DNA polymerase from *Pyrococcus* sp. strain KOD1 and its application to PCR. Appl. Environ. Microbiol. **63**, 4504–4510.
Uemori, T., Ishino, Y., Doi, H., and Kato, I. (1995) The hyperthermophilic archaeon *Pyrodictium occultum* has two α-like DNA polymerases. J. Bacteriol. **177**, 2164–2177.
Uemori, T., Ishino, Y., Toh, H., Asada, K., and Kato, I. (1993) Organization and nucleotide sequence of the DNA polymerase gene from the archaeon *Pyrococcus furiosus*. Nucleic Acids Res. **21**, 259–265.
Uemori, T., Sato, Y., Kato, I., Doi, H., and Ishino, Y. (1997) A novel DNA polymerase in the hyperthermophilic archaeon, *Pyrococcus furiosus*: gene cloning, expression, and characterization. Genes Cells **2**, 499–512.
Waga, S., and Stillman, B. (1998) The DNA replication fork in eukaryotic cells. Annu. Rev. Biochem. **67**, 721–51.
Wang, J., Sattar, A. K., Wang, C. C., Karam, J. D., Konigsberg, W. H., and Steitz, T. A. (1997) Crystal structure of α pol alpha family replication DNA polymerase from bacteriophage RB69. Cell **89**, 1087–1099.
Warbick, E. (1998). PCNA binding through a conserved motif. BioEssays **20**, 195–199.
Wernette, C. M., and Kaguni, L. S. (1986) A mitochondrial DNA polymerase from embryos of *Drosophila melanogaster*. J. Biol. Chem. **261**, 14764–14770.
Woese, C. R., and Fox, G. E. (1977) Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc. Natl. Acad. Sci. USA **74**, 5088–5090.
Woese, C. R., Kandler, O., and Wheelis, M. L. (1990) Towards a natural system of organisms: proposal for the domains archaea, bacteria, and eukarya. Proc. Natl. Acad. Sci. USA **87**, 4576–4579
Wong, S. W., Wahl, A. F., Yuan, P. M., Arai, N., Pearson, B. E., Arai, K.-I., Korn, D., Hunkapiller, M. W., and Wang, T. S.-F. (1988) Human DNA polymerase α gene expression is cell proliferation dependent and its primary structure is similar to both prokaryotic and eukaryotic replicative DNA polymerases. EMBO J. **7**, 37–47.
Xu, M.-Q., Southworth, M. W., Mersha, F. B., Hornstra, L. J., and Perler, F. B. (1993) *In vitro* protein splicing of purified precursor and the identification of a branched intermediate. Cell **75**, 1371–1377.
Zabel, H.-P., Fischer, H., Holler, E., and Winter, J. (1985) *In vivo* and *in vitro* evidence for eucaryotic α-type DNA polymerases in Methanogens. Purification of the DNA polymerase of *Methanococcus vannielii*. Syst. Appl. Microbiol. **6**, 111–118.
Zhou, M., Mao, C., Rodriguez, A. C., Kiefer, J. R., Kucera, R. B., and Beese, L. S. (1998) Crystallization and preliminary diffraction analysis of a hyperthermostable DNA polymerase from a *Thermococcus* archaeon. Acta Crystallogr. Sect. **D54**, 994–995.